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RESEARCH

Pollen Viability and Longevity of Switchgrass (*Panicum virgatum* L.)

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ABSTRACT

Pollen is essential for seed production and serves as the primary means of gene flow in outcrossing species like switchgrass (*Panicum virgatum* L.). There is a lack of information on basic pollen biology in switchgrass. This study investigated pollen viability, pollen longevity, and pollen size using different materials, including the tetraploid cultivar Alamo, the octoploid cultivar Cave-in-Rock, and transgenic Alamo plants. Pollen grains were collected from field-grown Alamo and Cave-in-Rock plants, and greenhouse-grown transgenics. Pollen size was in the range of 42.5 to 54.0 μm; no significant difference was observed in average pollen size between transgenic and control plants. Increasing temperature and ultraviolet-B irradiation negatively affected pollen viability and longevity, while relative humidity had only limited impact. Weather conditions had a large impact on pollen longevity. Under sunny atmospheric conditions, pollen longevity of both cultivars decreased rapidly, with a half-life of <4.9 min and a complete loss of viability in 20 min. Under cloudy atmospheric conditions, the half-life of pollen was more than fivefold longer than under sunny conditions, and it took approximately 150 min to lose viability completely. No difference in pollen viability and longevity was found between transgenic and nontransgenic control plants.

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Abbreviations: RH, relative humidity; UV-B, ultraviolet-B.

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial bunchgrass native to North America. Being one of the predominant species found in tallgrass prairies, switchgrass has traditionally been used for soil conservation and forage production (Bouton, 2007; Vogel and Jung, 2001). Because of its high biomass productivity, low nutrient requirement, and potential environmental benefits, switchgrass has been developed into a major herbaceous bioenergy crop for the production of cellulosic biofuels in recent years (Hisano et al., 2009; McLaughlin et al., 2006; Schmer et al., 2008).

An important aspect of switchgrass taxonomy and reproductive behavior is its grouping into two distinct ecotype categories: lowland and upland (Bouton, 2007; Shen et al., 2009). Lowland switchgrass plants are taller, coarser, and more productive than uplands, while uplands have longer rhizomes and are able to grow in drier, colder zones. Lowlands are predominately tetraploids ($2n = 4x = 36$), whereas the majority of upland switchgrass is octoploid ($2n = 8x = 72$) (Bouton, 2007).

The development of transgenic switchgrass has great potential to improve biofuel production efficiency. For example, a recent study has shown that downregulation of the caffeic acid *O*-methyltransferase gene decreases lignin content modestly and increases ethanol yields by up to 38% (Fu et al., 2011a). The downregulated

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lines require reduced pretreatment severity and 300 to 400% lower cellulase dosages for equivalent product yields (Fu et al., 2011a). Although the improvement is large and the approach is straightforward, commercialization of transgenic perennial biofuel crops is complicated because of strict regulatory restrictions (Strauss et al., 2010).

Switchgrass is a wind-pollinated species with a high degree of self-incompatibility. Because wind-pollinated grasses may easily pass their genes to adjacent populations, pollen-mediated gene flow is an important issue for field release of transgenic grasses (Wang and Ge, 2006). Pollen viability and longevity is one of the key factors in risk assessment and transgene containment of switchgrass.

Several staining methods have been developed to evaluate pollen viability, such as the use of iodine staining to determine starch content, tetrazolium salts to detect dehydrogenase activity, aniline blue to detect callose in pollen walls and pollen tubes, and fluorescein diacetate to determine esterase activity and the intactness of the plasma membrane (Shivanna et al., 1991; Wang et al., 2004). Different staining methods were tested in tall fescue (*Festuca arundinacea* Schreb.) and other grasses (e.g., meadow fescue [*Festuca pratensis* Huds.] and Russian wildrye [*Psathyrostachys juncea* (Fisch.) Nevski]) (Wang et al., 1993, 2002, 2004). Although these methods offered a quick way of detecting aborted and nonaborted fresh pollen, they failed to distinguish between viable pollen and dead pollen (Wang et al., 2004). By optimizing composition of a pollen germination medium, a simple in vitro germination protocol was developed to effectively assess pollen viability in tall fescue (Wang et al., 2004).

Knowledge about the duration of pollen viability as well as pollen size provides baseline information for developing ways to manage pollen flow or devising methods for transgene containment. To the best of our knowledge, there have been no reports on pollen viability and longevity in switchgrass. The objectives of this study were to (i) compare pollen size, viability, and longevity of nontransgenic and transgenic switchgrass; and (ii) evaluate the effects of different environmental variables (temperature, humidity, ultraviolet-B [UV-B] treatment) on pollen viability and longevity.

MATERIALS AND METHODS

Plant Materials

A lowland switchgrass cultivar, Alamo ($2n = 4x = 36$), and an upland cultivar, Cave-in-Rock ($2n = 8x = 72$), were used in the study. Alamo is the most widely grown switchgrass cultivar, and transgenic plants have been produced from this cultivar in several laboratories. Cave-in-Rock is known for high palatability and high biomass potential compared with other upland cultivars. Seed-derived plants were grown in the experimental field in Ardmore, OK. The materials were used for pollen collection and subsequent measurement of pollen size, and assessment of pollen viability and longevity.

Transgenic switchgrass plants were obtained by *Agrobacterium*-mediated transformation of embryogenic calli (Fu et al., 2011a; Xi et al., 2009). An RNAi construct of the caffeic acid 3-*O*-methyltransferase (*COMT*) gene was introduced into switchgrass cv. Alamo. Molecular and biochemical analyses identified transgenics with reduced lignin, altered lignin composition, increased saccharification efficiency, and improved ethanol yield (Fu et al., 2011a). Selected transgenics were crossed with a nontransgenic plant. Both COMT RNAi-positive and -negative (null segregant) plants were identified from the progeny of each cross (Fu et al., 2011a), and the negative plants were used as controls for pollen measurements of the T_1 transgenics. Transgenic T_1 plants and corresponding controls were grown in the greenhouse.

For pollen collection, flowering inflorescences were harvested in the morning and brought to the laboratory. Lower parts of the inflorescences were placed in water and the inflorescences were stored in a growth chamber at 24°C. Pollen grains were normally collected 3 to 4 h after field harvesting of the inflorescences and subjected to different treatments immediately. Although pollen can be directly collected from materials in the field, it takes time to travel from the field to the laboratory and some pollen may lose their viability during this period. In addition, because of wind and rapid anther dehiscence, it is difficult to collect large amounts of pollen at exactly the same time in the field.

Test of Pollen Viability by Staining

Dead pollen was obtained by treatment at 80°C for 2 h. Lugol staining and aniline blue staining were tested for fresh and dead pollen. The Lugol solution (Sigma, St. Louis, MO) consists of iodine and potassium iodide. The method detects starch content; black-stained pollen was considered viable. Aniline blue detects callose in pollen walls and pollen tubes (Hauser and Morrison, 1964; Khatun and Flowers, 1995). The aniline blue–lactophenol staining solution was made by adding 5 mL of 1% (v/v) aqueous aniline blue to a medium of 20 mL of phenol, 20 mL of lactic acid (ca. 85% [v/v]), 40 mL of glycerine, and 15 mL of H_2O.

Assessment of Pollen Viability and Longevity by In Vitro Germination

The pollen germination medium developed for tall fescue (Wang et al., 2004) was used for assessing viability of switchgrass pollen. The simple germination medium is composed of 1% agar, 0.8 M sucrose, 1.28 mM boric acid, and 1.27 mM calcium nitrate. Bulk pollen was distributed onto germination media in petri dishes and incubated at 24°C for 4 h. Germination was quantified as the percentage of germinated pollen grains per 100 evaluated. Pollen grains were considered germinated when the pollen tube length was greater than the diameter of the pollen grain (Tuinstra and Wedel, 2000). To measure pollen size, pollen grains were distributed onto germination medium and their sizes were immediately measured using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan).

Effects of Different Conditions on Pollen Viability and Longevity

To test the effect of temperature, pollen was incubated for up to 60 min at 18, 20, 24, 28, 32, 36, 40, and 44°C before being dispensed onto the germination medium. The temperature treatment was performed in ABI Veriti Thermal Cyclers (Applied

Biosystems, Carlsbad, CA), which provided precise and immediate temperature controls. The effect of RH was tested after storing pollen at 40, 60, 80, and 99% RH in bioclimatic chambers (model E-54U, Percival Scientific, Perry, IA) for 10 min and 20 min at 24°C before being dispensed onto the germination medium. Effect of ultraviolet irradiation was performed by exposing pollen to UV-B light (15w UV-B lamps, UVP Inc., Upland, CA) fixed on an exposure stand for 10 min before pollen grains were germinated on the medium at 24°C. The UV-B doses were 300, 600, 900, 1200, and 1500 μW cm^{-2}, determined by UVX digital radiometer with UV-B-specific sensor UVX-31 (UVP Inc., Upland, CA). The dosage used in the experiment was in the range that plants may encounter under natural conditions.

Pollen longevity under natural conditions (sunny and cloudy) was tested by exposing pollen to outdoor open air. Under sunny conditions, the open air temperature was about 32°C and RH was at 46%. Under cloudy conditions, the air temperature was about 27°C and RH was 61%. Pollen longevity under greenhouse conditions was also tested by placing collected pollen grains in a greenhouse room without strict climate control. The temperature of the greenhouse room reached 39°C under sunny conditions and 30°C on a cloudy day. The humidity levels in the greenhouse were 84.8 and 85.7% under sunny and cloudy conditions, respectively.

Comparison of Pollen Viability and Longevity in Transgenic and Control Plants

Pollen was collected from greenhouse-grown transgenic and nontransgenic control plants. Pollen viability was evaluated at different time points.

Statistical Analysis

Each experiment described above was performed in a randomized complete block design with three replications, where each collection of inflorescences was considered as one replication (i.e., a block). Data were analyzed using ANOVA and all the treatment differences were computed at $P < 0.05$. Standard errors are provided in the figures.

To calculate the half-life of switchgrass pollen, an exponential model was established to describe the relationship between the time and percentage of pollen germination under different treatments. Pollen germination data were collected and fitted with an exponential program using OriginPro 8.5 (OriginLab Corporation, Northampton, MA). The half-life ($t_{1/2}$) of pollen was obtained through computation with the parameters generated by nonlinear curve fitting.

RESULTS

Size of Switchgrass Pollen

Diameter of pollen collected from field-grown plants was measured using a microscope. Pollen size of the tetraploid cultivar Alamo was in the range of 44.0 to 52.0 μm, with an average size of 48.4 ± 0.3 μm. Pollen size of the octoploid cultivar Cave-in-Rock varied from 44.5 to 54.0 μm, with an average size of 51.2 ± 0.4 μm. The size of Cave-in Rock pollen is larger than that of Alamo.

Pollen grains were also collected from transgenic and corresponding control Alamo plants grown in the greenhouse. Pollen size from transgenic plants was in the range of 42.5 to 51.5 μm, with an average size of 46.6 ± 0.5 μm. Pollen size from control plants varied from 43.0 to 51.0 μm, with an average size of 47.2 ± 0.4 μm. No significant difference was observed between pollen from transgenic and control plants.

Viability Test and In Vitro Germination of Switchgrass Pollen

Switchgrass pollen could be easily stained with aniline blue and Lugol solution (data not shown); however, none of the methods could detect the difference between fresh and dead (treated) pollen. The results are similar to that of tall fescue (Wang et al., 2004) and relate to the mechanisms of the staining methods. For example, Lugol solution stains starch, and starch still remains in the pollen and stainable after pollen was killed at 80°C for 2 h.

Switchgrass pollen placed onto a simple germination medium started to germinate in about 5 min, and pollen viability could be assessed after 20 min. Figure 1 illustrates the germination process of switchgrass pollen. The method offers a quick and reliable way of evaluating pollen viability and longevity in switchgrass. No dead pollen was able to germinate on the medium.

Effect of Temperature on Pollen Viability and Longevity

Pollen germination in Alamo and Cave-in-Rock was evaluated over a range of temperatures that might be encountered under field conditions. As shown in Fig. 2, pollen viability dropped rapidly with increasing temperature. After treatment at various temperatures (18–40°C), pollen viability of the cultivar Alamo (Fig. 2A) declined faster than that of Cave-in-Rock (Fig. 2B). For example, after 60 min at 18°C and 24°C, the remaining pollen viability of Alamo was only 2.3 and 2.0%, respectively, whereas 20.0 and 7.6% of Cave-in-Rock pollen were still viable. At the highest temperature (44°C) tested in the experiment, pollen viability of both cultivars dropped to zero after 10 min.

Pollen half-life ($t_{1/2}$) of Alamo decreased from 28.7 min at 18°C to 9.3 min at 32°C (Fig. 2A). Pollen half-life of Cave-in-Rock decreased from 40.4 min at 18°C to 9.5 min at 32°C (Fig. 2B). When temperature was higher than 36°C, there were too few data points to calculate the half-life of pollen (Fig. 2A and 2B). At relatively low temperatures (18–24°C), the half-life of Cave-in-Rock pollen was much longer (>11.2 min) than that of Alamo pollen. When the temperature was higher than 28°C, the difference between the half-lives of the two cultivars was much smaller (<1.9 min) (Fig. 2A and 2B).

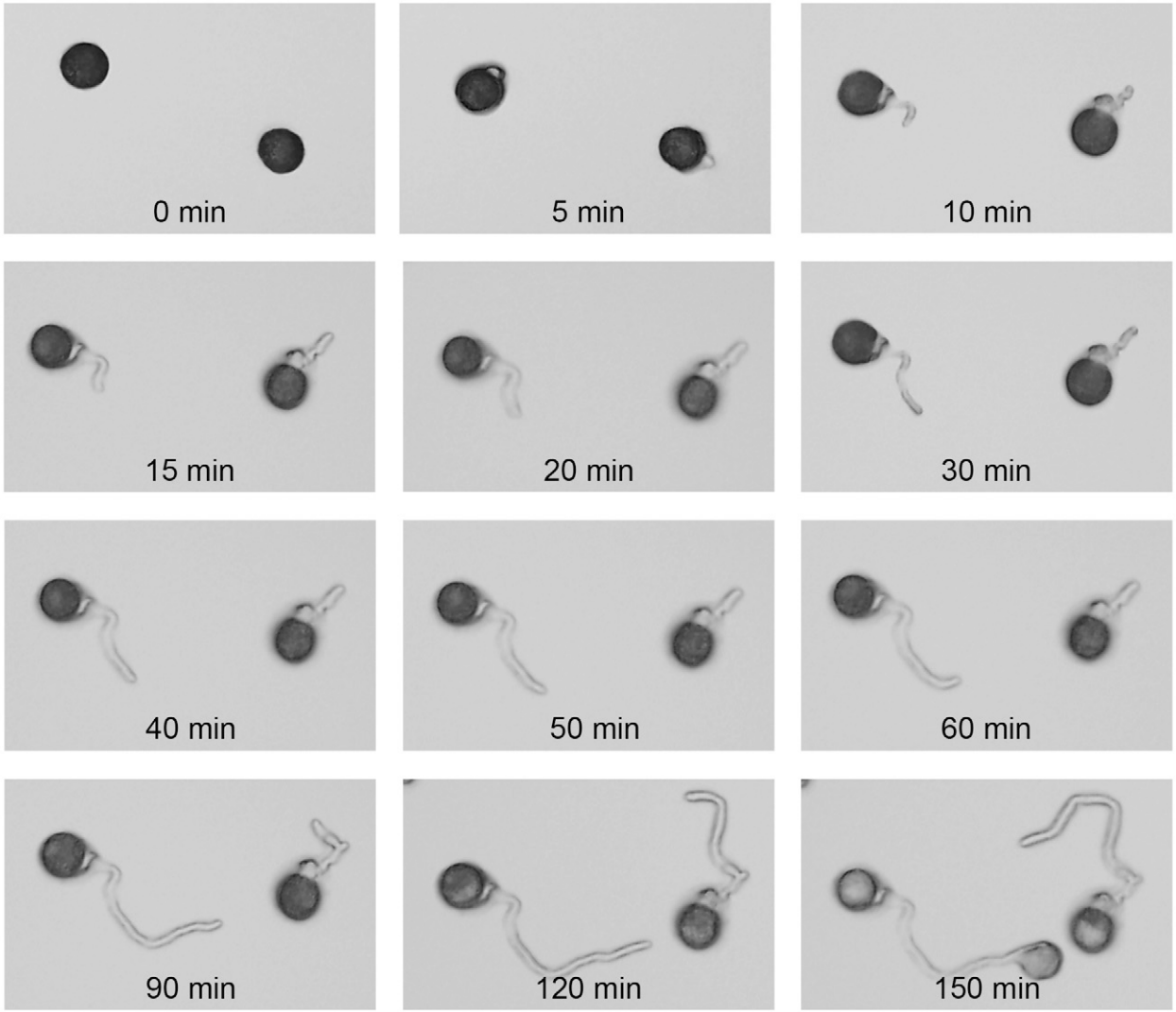

Figure 1. Germination process of switchgrass (cv. Alamo) pollen on a medium consisting of 1% agar, 0.8 M sucrose, 1.28 mM boric acid, and 1.27 mM calcium nitrate.

Effect of Humidity and Ultraviolet-B Irradiation on Pollen Viability

Pollen viability was tested after storing pollen at different relative humidity levels. For Alamo, significantly reduced viability was observed when pollen was incubated at 40% RH, particularly when incubation time was 20 min. No difference was found when pollen was incubated at 60, 80, and 99% RH (Fig. 3A). For Cave-in-Rock, significant difference in pollen viability was only observed between 80 and 99% RH after 10 min incubation. After 20 min incubation, relatively high pollen viability was observed at 60 and 80% RH (Fig. 3B).

Switchgrass pollen was subjected to different doses of UV-B treatments. With the exception that no difference was detected between 1200 and 1500 $\mu W\ cm^{-2}$ treatment after 20 min, pollen viability was inhibited with increasing doses of UV-B irradiation (Fig. 4). The trend was similar between Alamo and Cave-in-Rock (Fig. 4).

Longevity of Switchgrass Pollen under Greenhouse and Ambient Atmospheric Conditions

Viability of switchgrass pollen was tested in the greenhouse and in outside open air under sunny (direct sunlight) and cloudy conditions (Fig. 5). A rapid decrease in pollen viability was observed for both cultivars under sunny conditions. Less than 5% of pollen remained viable after 9 min, and all the pollen lost their viability after 20 min under the sun (Fig. 5A). The pollen half-life of Alamo and Cave-in-Rock under sunny conditions was 4.3 and 4.9 min, respectively. Because of the rising temperature in the greenhouse during a sunny day, pollen half-life in the greenhouse was reduced for Alamo (3.7 min) and extended for Cave-in-Rock (5.2 min) when compared with that under direct sunlight (Fig. 5A).

Under cloudy conditions, pollen remains viable for a longer period of time, and the decrease in pollen viability was

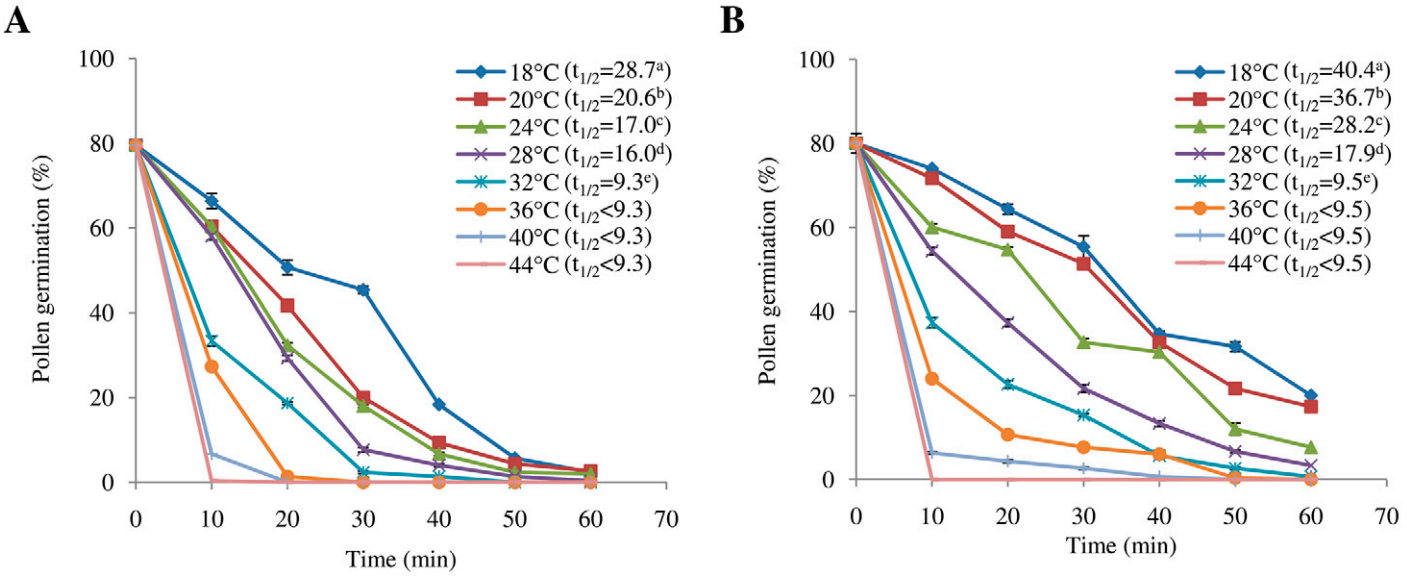

Figure 2. Effect of temperature on pollen viability and longevity in (A) lowland cv. Alamo and (B) upland cv. Cave-in-Rock. $t_{1/2}$: pollen half-life. $t_{1/2}$ values with the same letters are not significantly different at p = 0.05 within the cultivar. Vertical bars represent standard errors at different time points.

much less dramatic than under sunny conditions (Fig. 5B). Pollen half-life of Alamo and Cave-in-Rock under cloudy conditions was 22.9 and 28.2 min, respectively, which was 5.3- to 5.8-fold longer than corresponding values under sunny conditions. It took about 150 min for all the pollen to lose their viability (Fig. 5B). In the greenhouse during a cloudy day, pollen half-life of Alamo and Cave-in-Rock was 14.1 and 19.7 min, respectively (Fig. 5B).

Comparison of Pollen Viability of Transgenic and Nontransgenic Plants

Pollen collected from transgenic and nontransgenic switchgrass (cv. Alamo) plants grown in the greenhouse did not show any significant difference in viability and longevity (Fig. 6).

DISCUSSION

To grow transgenic switchgrass in the greenhouse, air filters are required to block pollen escape. Therefore, information regarding pollen size is essential for choosing the correct filter sizes. In this study, no significant difference was found in average pollen size between transgenic and nontransgenic plants, and their pollen size ranges were very close. The diameter of viable pollen of Alamo varied from 42.5 to 52.0 μm, whereas some empty or aborted pollen were smaller in size. Filters with sufficiently small pore size (e.g., <10 μm) should be adequate to capture

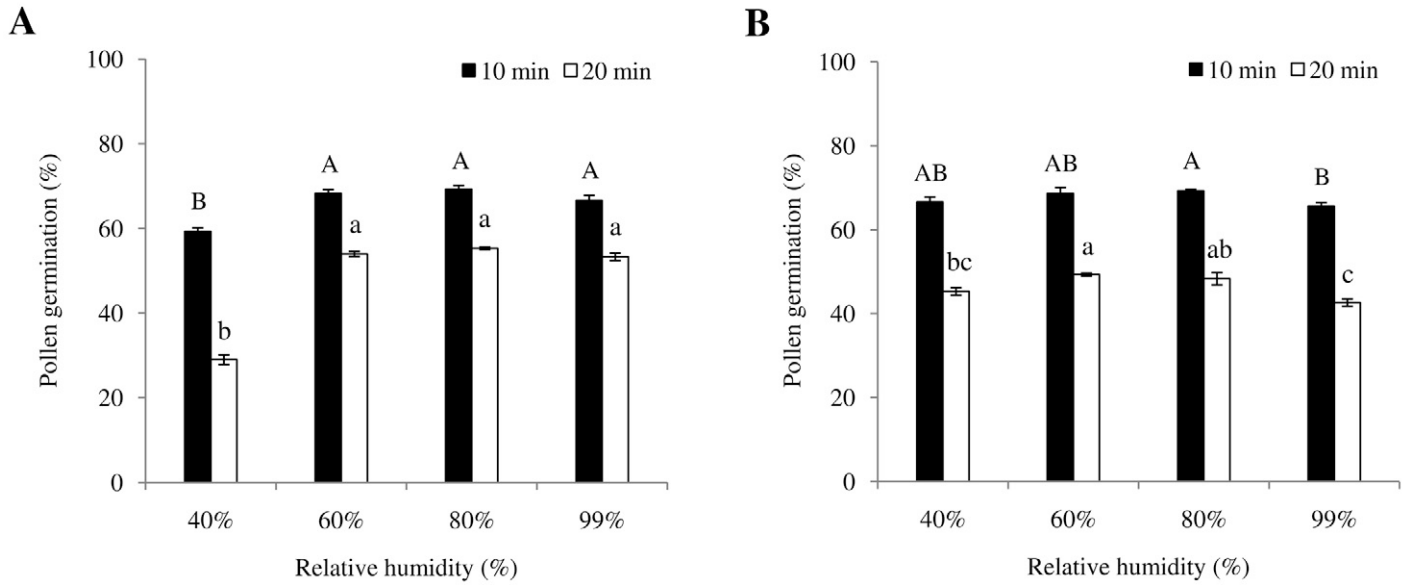

Figure 3. Effect of relative humidity on pollen viability in (A) lowland cv. Alamo and (B) upland cv. Cave-in-Rock. Columns labeled with the same letters (uppercase letters for 10-min treatment, lowercase letters for 20-min treatment) are not significantly different at p = 0.05. Vertical bars at the top of the columns represent standard error of the means.

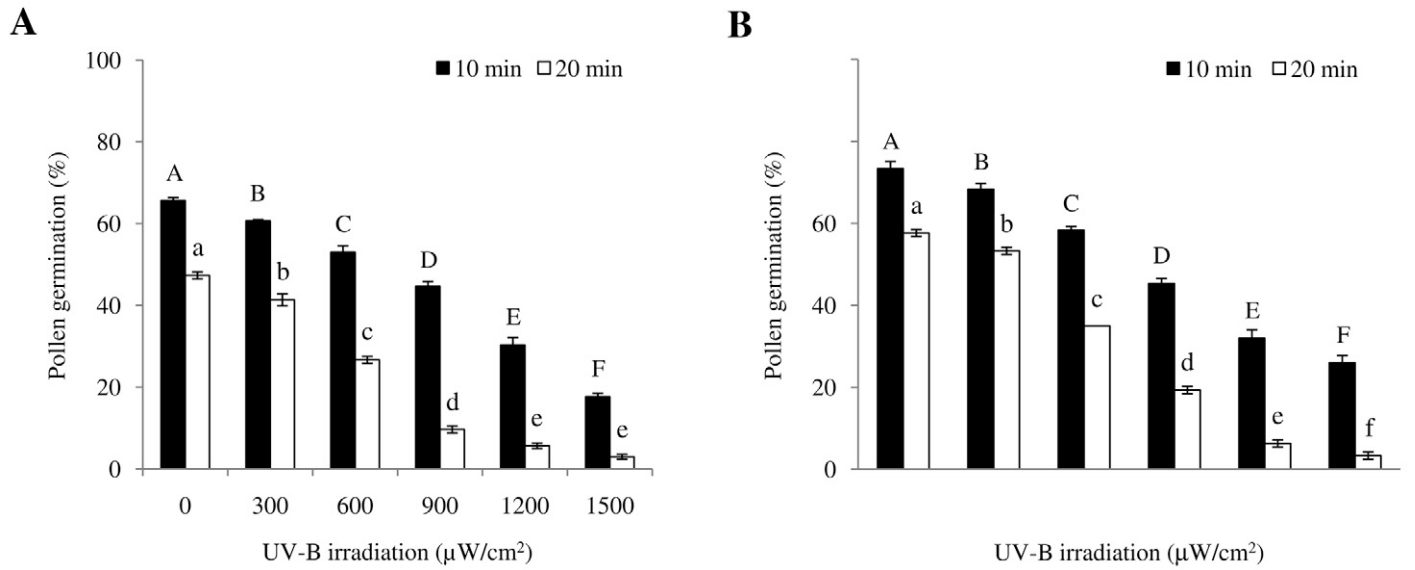

Figure 4. Effect of ultraviolet-B (UV-B) irradiation on pollen viability in (A) lowland cv. Alamo and (B) upland cv. Cave-in-Rock. Columns labeled with the same letters (uppercase letters for 10-min treatment, lowercase letters for 20-min treatment) are not significantly different at $p = 0.05$. Vertical bars at the top of the columns represent standard error of the means.

viable switchgrass pollen. Pollen size of the octoploid cultivar Cave-in Rock is larger than that of the tetraploid cultivar Alamo, probably due to the higher ploidy level.

In vitro pollen germination has been considered the most effective method of estimating pollen viability in vivo (Stone et al., 1995; Tuinstra and Wedel, 2000). The staining methods (aniline blue, Lugol solution) failed to distinguish viable and dead pollen grains of switchgrass. These staining methods are thus not suitable for the study of pollen viability and longevity in switchgrass. The simple germination medium developed for tall fescue proved to be suitable for analyzing pollen viability in switchgrass.

Temperature is one of the key environmental factors that can vary considerably during the flowering period of switchgrass. In all cases, switchgrass pollen viability and longevity decrease with increasing temperature. The result is somewhat different from that of tall fescue, in which pollen viability was only reduced when temperature was at or above 36°C (Wang et al., 2004). In switchgrass, high temperatures result in rapid decrease in pollen viability. The difference between the lowland cultivar Alamo and the upland cultivar Cave-in-Rock in their response to temperature is noteworthy. At a relatively low temperature (18–24°C), the half-life of Cave-in-Rock pollen was much longer than that of Alamo. Although Cave-in-Rock still had a longer half-life at higher temperatures (28–44°C), the differences between the two cultivars were much smaller. Such temperature response may reflect the adaptability or

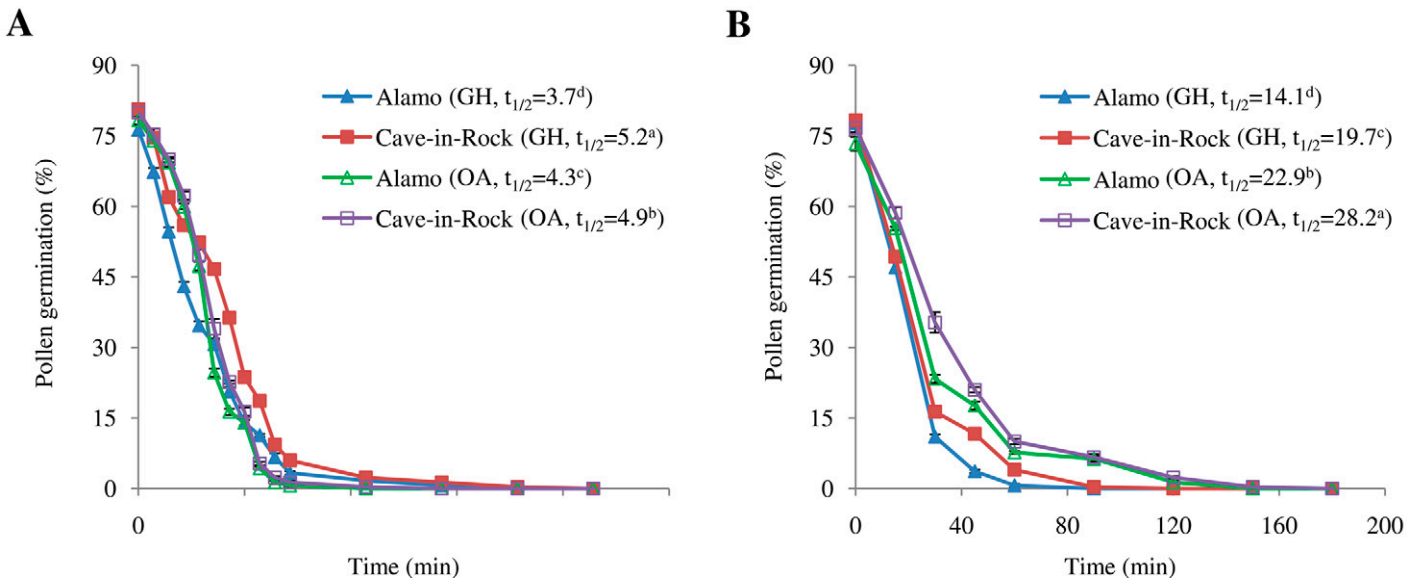

Figure 5. Pollen longevity of lowland cv. Alamo and upland cv. Cave-in-Rock under (A) sunny and (B) cloudy conditions. GH: in greenhouse; OA: in outside open air; $t_{1/2}$: pollen half-life. $t_{1/2}$ values with the same lowercase letters are not significantly different at $p = 0.05$. Vertical bars represent standard errors at different time points.

fitness difference between the two types of cultivars. The ability of pollen to remain viable for a longer time under low temperature offers an advantage for seed production of upland cultivars in colder regions.

Relative humidity is another environmental factor that might affect pollen viability. Pollen from graminaceous species require high relative humidity, although low relative humidity is favorable for pollen storage in many other species (Adhikari and Campbell, 1998). In the present study, pollen viability of Alamo was significantly reduced at low relative humidity (RH 40%), particularly after incubation at this humidity level for 20 min. Reduced pollen viability was observed for Cave-in-Rock at 99% RH. Besides the effects of temperature and humidity, pollen viability was reduced with increasing UV-B doses, indicating ultraviolet irradiation may contribute to reduced pollen longevity under direct sunlight.

Longevity of switchgrass pollen was influenced drastically by weather conditions. Under sunny atmospheric conditions, there was a rapid reduction in pollen viability for both cultivars, with a half-life of <4.9 min and a complete loss of viability in 20 min. A similar trend was observed when pollen was placed in the greenhouse. Although ultraviolet irradiation was largely eliminated, temperature in the greenhouse room increased on a sunny day. Under cloudy atmospheric conditions, the half-life of pollen was more than fivefold longer than that under sunny conditions, and it took approximately 150 min to lose viability completely. Longevity of pollen has been reported in other grass species. For example, viability of tall fescue pollen was completely lost after 90 and 240 min under sunny and cloudy conditions, respectively (Wang et al., 2004); no viable pollen was detected within 70 min in wheat (*Triticum aestivum* L.), and 120 min in triticale (*Triticale hexaploide* Lart.) (Fritz and Lukaszewski, 1989) and maize (*Zea mays* L.) (Luna et al., 2001).

Evaluation of viability and longevity of transgenic pollen is an important aspect of risk assessment for transgenic plants (Wang and Ge, 2006; Wang et al., 2004). No difference in pollen viability and longevity was found between transgenic and nontransgenic control plants, indicating that the knowledge obtained from the study of nontransgenic pollen can be applied to transgenic pollen. The COMT downregulated transgenics were phenotypically normal in the greenhouse (Fu et al., 2011a). The results on pollen viability further confirm that pollen development was normal in COMT downregulated transgenics.

Genetic engineering has greatly contributed to breakthroughs in plant improvement and is expected to play an important role in biofuel production by modifying the quantity or quality of biomass (Gressel, 2008; Hisano et al., 2009). In recent years, progress has been made in developing transformation techniques and producing transgenic switchgrass with improved biomass traits (Burris et al., 2009; Fu et al., 2011a, 2011b; Li and Qu, 2011; Somleva et al., 2008; Xi et al., 2009). Because the product is not for human consumption, risk assessment of transgenic switchgrass will likely focus on its environmental or ecological impacts, in which pollination biology plays a critical role. This is the first report on assessing switchgrass pollen viability and longevity using different materials and under different conditions. The baseline information will be useful for breeding and biosafety studies of transgenic bioenergy crops.

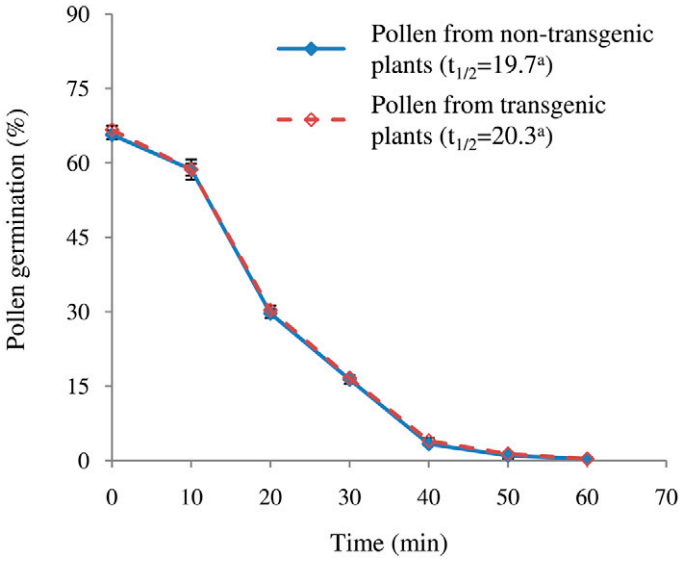

Figure 6. Pollen viability and longevity of greenhouse-grown transgenic and nontransgenic switchgrass (cv. Alamo). $t_{1/2}$: pollen half-life. $t_{1/2}$ values with the same letters are not significantly different at p = 0.05. Vertical bars represent standard errors at different time points.

Acknowledgments

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